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Influence of detergents on the activity of the ABC transporter LmrA

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ABSTRACT

The ABC transporter LmrA from *Lactococcus lactis* has been intensively studied and a role in multidrug resistance was proposed. Here, we performed a comprehensive detergent screen to analyze the impact of detergents for a successful solubilization, purification and retention of functional properties of this ABC transporter. Our screen revealed the preference of LmrA for zwitterionic detergents. In detergent solution, LmrA purified with FC-16 was highly active with respect to ATPase activity, which could be stimulated by a substrate (rhodamine 123) of LmrA. Both, high ATPase activity and substrate stimulation were not detected for LmrA solubilized in DDM. Interestingly, reconstituted LmrA showed an opposite behavior, with a high basal ATPase activity and stimulation by rhodamine 123 for a DDM-reconstituted, but only low ATPase activity and no substrate stimulation for a FC-16 reconstituted sample.

1. Introduction

Modern biochemistry provides researchers with a wide range of techniques to study proteins in detail and to investigate their mode of action. Some of the most powerful techniques to analyze the structure as well as the mechanism of proteins can only be applied *in vitro*. This obviously requires purification of the protein of interest. For soluble proteins this is normally straightforward, while it often remains a challenging task for membrane proteins. Two of the most critical factors that determine the success of membrane protein purification are the level of expression and the choice of detergent. While a wealth of information is available in the literature (for example [1–4]) concerning the expression of membrane proteins, only a small number of publications exist that deal with the role of detergents in membrane protein purification (e.g. [5–9]). Nevertheless detergents are key players in the purification of membrane proteins, because these proteins are attached to or integrated into a biological membrane, which usually makes it necessary to solubilize them from the membrane with detergents for subsequent purification. Therefore, the selection of a suitable detergent is an essential step in membrane protein purification, because it not only has to ensure efficient solubilization but also has to stabilize the protein in order to retain its structure and function. Unfortunately, the choice of detergent is not trivial, because a multitude of possibly useful detergents can be used but only a limited amount of data are available concerning their effect on membrane proteins. For the study of membrane proteins this means that a detergent that works best for all membrane proteins does not exist [10], although detergents such as dodecyl-β-D-maltoside (DDM) or octyl-β-D-glucopyranosid proved to be successful for the solubilization of many membrane proteins [11,12]. Therefore, the first step is a detergent screen with a more or less limited number of detergents and the analysis of the efficiency of the solubilization process (e.g. by western blotting) in combination with an examination of functional and/or structural properties of the (purified) protein [13–17].

We decided to use a membrane protein that can be highly overexpressed in its original host for our investigation. LmrA from *Lactococcus lactis* fulfils this demand and belongs to the class of ATP-binding cassette (ABC) transporters. Among membrane proteins, ABC transporters form one of the most ubiquitously distributed protein families [18]. Most members of this protein family use the energy of ATP-hydrolysis to transport their substrates across a biological membrane against a concentration gradient [19,20]. All ABC-transporters described so far, share a common core domain organisation, in which a functional transporter consists of two nucleotide binding domains (NBDs) that energize transport through the binding and hydrolysis of ATP and two transmembrane domains (TMDs) that are thought to form the pathway for substrate translocation. However, individual ABC transporters may require further domains [21–23] such as the regulatory domain of CFTR [24] or the substrate binding proteins of ABC importers [25]. LmrA is an ABC-exporter that contains one TMD with six predicted α-helices fused to one NBD, an arrangement referred to as "half-size" transporter. The functional unit of LmrA is thought to be

Abbreviations: DDM, dodecyl-β-D-maltoside; FC-16, FOS-CHOLINE-16; MDR, multidrug resistance; SEC, size exclusion chromatography; $C_{10}E_5$, Pentaethylene glycol monodecyl ether

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a homodimer [26]. LmrA shares 34% sequence identity and an additional 14% of conservative substitutions with the human P-glycoprotein (Pgp, MDR1 or ABCB1) the archetype of a multidrug resistance (MDR) ABC-transporter [27–29]. In addition, the expression of LmrA in human lung fibroblasts was reported to confer a MDR phenotype with cells expressing LmrA displaying an increased resistance to Pgp substrates, which can be affected by several Pgp modulators [30].

To shed light on the influence of detergents on the functional and structural properties of ABC-transporters, we solubilized and purified LmrA with 40 different detergents. All set-ups were analyzed at different time points by means of SEC and ATPase activity to study the oligomeric state, stability and homogeneity of LmrA as well as its ability to hydrolyze ATP. For the most successful detergents FOS-CHOLINE-16 (FC-16) and dodecyl-β-D-maltoside (DDM) the kinetics of ATP hydrolysis of LmrA was further analyzed in the solubilized state as well as after reconstitution into *Escherichia coli* polar lipid-based liposomes. These experiments provided important insights into the effect of detergents on the functional properties of LmrA, which was not anticipated before.

2. Materials and methods

2.1. Materials

M17 medium components were obtained from Carl Roth; all other chemicals were purchased from Sigma-Aldrich; detergents were obtained from Anatrace; BioBeads SM2 and *E. coli* total lipid extract were from Bio-Rad Laboratories Inc. and Avanti Polar Lipids, respectively. For determination of the protein concentration, a Coomassie Plus Assay (Pierce) was used.

2.2. Expression

L. lactis strain NZ 9000 Δ*lmrA* Δ*lmrCD* harbouring the plasmid pNHLmrA was cultivated at 30 °C in M17 medium supplemented with 0.5% (wt/v) glucose and 5 μg/ml chloramphenicol. Cells were grown to an OD_{660} of about 0.8 and expression of LmrA was induced by addition of 25 μg/l Nisin (Fluka). After 2 h of expression cells were harvested and stored at −80 °C.

2.3. Purification of LmrA

The cell-suspension was thawed and incubated with lysozyme (10 mg/ml) for 30 min at 30 °C. Subsequently the suspension was cooled on ice for 15 min and EDTA was added to a final concentration of 4 mM. Cells were lysed by passing them four times through a cooled TS Series Cell Disruptor (Constant Systems) at 2.5 kbar. Cell debris and intact cells were removed by two centrifugation steps (45 min at 13,000*g*, 30 min at 20,000 g, 4 °C). Membrane vesicles were collected by ultracentrifugation for 1 h at 125,000 g, 4 °C. The membrane vesicles were suspended in buffer 1 (50 mM Hepes pH 8, 10 mM imidazole, 250 mM NaCl and 10% (v/v) glycerol) and the protein concentration was determined with a Coomassie Plus Assay (Pierce) according to manufacturer recommendations. Membrane vesicles were solubilized at a protein concentration of 15 mg/ml with 1% detergent for 30 min at 4 °C if not stated otherwise (see Table 1). Insoluble material was removed by ultracentrifugation for 30 min at 125,000*g*, 4 °C. The supernatant was loaded onto a zinc-loaded HiTrap Chelating column (1 ml, GE Healthcare) and washed with buffer 2 (50 mM Hepes pH 7, 40 mM imidazole, 250 mM NaCl, 10% (v/v) glycerol) supplemented with typically 2.5 times cmc of detergent (see Table 1) until a stable baseline was reached. His-tagged LmrA was eluted with buffer 3 (50 mM Hepes pH 7, 250 mM imidazole, 250 mM NaCl, 10% (v/v) glycerol) supplemented with detergent (see Table 1). Purified LmrA was directly used for reconstitution, measurement of ATPase activity and SEC. For further characterization protein samples were stored at 4 °C and analyzed again after 1 week of storage. The purity of the protein preparation was assessed by SDS-PAGE.

2.4. Size exclusion chromatography (SEC)

SEC was employed to assess the homogeneity of the different LmrA preparations. In brief, SEC was performed on a SMART System with a Superose 12 3.2/30 column (GE Healthcare) at 4 °C with a flow rate of 50 μl/min using buffer 4 (20 mM Hepes pH 7, 100 mM NaCl, 10% (v/v) glycerol) supplemented with detergent (typically at a concentration of 2.5 times cmc; see Table 1). To calibrate the Superose 12 column several soluble standard proteins (Sigma-Aldrich) of known molecular weight were injected as described above, but without the addition of detergent to buffer 4.

2.5. Reconstitution of LmrA into proteoliposomes

Large unilamellar vesicles (LUVs) of *E. coli* total lipids were prepared in buffer 5 (50 mM Hepes, pH 7.4, 150 mM NaCl and 10% (v/v) glycerol) as described in Ref. [31] and destabilized with detergent [32] to allow insertion of protein. To determine the onset of solubilization (R_{sat}) where the liposomes are saturated with detergent as well as complete solubilization of the liposomes (R_{sol}) a titration of liposomes with detergent was performed as described in Ref. [31].

For a typical reconstitution of LmrA, 16 mg of LUV suspension at a concentration of 2 mg/ml were destabilized with detergent to a point close to R_{sat} for 30 min at 25 °C. Next purified protein was added to result in a protein to lipid ratio of 1:30 to 1:100 (wt/wt). Subsequently the samples were incubated for 2 h at 4 °C with gentle agitation, followed by the removal of the detergent by the adsorption to BioBeads. In a first step, 80 mg BioBeads per ml suspension were added and incubated with the sample at 4 °C with gentle agitation. After 1 h additional 100 mg of BioBeads per ml of suspension were added. After 40 h of incubation the Biobeads were removed and the samples were diluted ten-fold with buffer 5. The proteoliposomes were collected by ultracentrifugation for an hour at 125,000 g, 4 °C. The resulting pellets were suspended at a concentration of 8 mg/ml in buffer 5. Large particles were removed by an additional centrifugation step at 13,000g, 4 °C for 15 min.

2.6. Reconstitution efficiency

The amount of reconstituted protein in the proteoliposomes and the vesicle recovery was determined as described in Ref. [33]. A BCA protein assay (Pierce) was used for the determination of the protein concentration. The lipid concentration was assayed by measuring the total phosphorus in the sample.

2.7. Measurement of the ATPase activity

The ATPase activity of LmrA was monitored by the liberation of inorganic phosphate, which was quantified with molybdate/ malachite green as described [34,35]. The reactions were performed in duplicate in a total volume of 200 μl in buffer 4 supplemented with 5 mM $MgCl_2$ and where appropriate modulator and/or detergent (typically at a concentration of 2.5 times cmc; see Table 1). For the assays between 1 μg and 50 μg of protein were used, depending on the activity of the protein. The reaction was started by addition of ATP (typically 1 mM if not stated otherwise) and samples were incubated for a time period ranging from 30 min up to 2 h at 25 °C. At the indicated time points the reaction was stopped by transferring 25 μl of the sample into 175 μl 20 mM H_2SO_4. Subsequently 50 μl dye solution (0.096% (w/v) malachite green, 1.48% (w/v) ammonium molybdate, and 0.173% (w/v) Tween-20 in 2.36 M sulfuric acid) was added. After 15 min the amount of free phosphate was quantified spectroscopically by the absorption at 620 nm. For the measurements all appropriate controls were performed and subtracted

Table 1
Detergent concentrations employed for solubilization and purification of LmrA as well as the resulting protein yields.

Detergent	Concentration used for solubilization	Concentration used for purification	Times cmc	Protein yield (mg/5 l culture)
Dodecyl-β-D-glucopyranosid	1%	0.0165%	2.5	0.1
Decyl-β-D-glucopyranosid	1%	0.175%	2.5	0.1
Octyl-β-D-glucopyranosid	1%	1.06%	2.0	0.1
C-HEGA 11	1%	0.495%	1.1	0.1
FOS-MEA 10	1%	0.375%	2.5	0.1
FOS-MEA 12	1%	0.035%	2.5	0.1
Sodiumdodecanoylsarcosine	1%	0.42%	1.0	0.3
6-O-Methyl-n-heptylcarboxyl-α-D-glucopyranoside	1%	0.345%	1.5	0.1
CYGLU 4	1%	0.145%	2.5	0.2
Big CHAP deoxy	1%	0.24%	2.0	0.2
CTAB	1%	0.091%	2.5	n.a.
2-Carboxy-ω-heptadecenamidopropyldimethylamine	1%	0.00095%	2.5	2.5
Tetradecyl-β-D-maltopyranoside	1%	0.00135%	2.5	1.0
CHAPSO	2%	0.75%	1.5	1.3
SodiumCholate	2%	0.41%	1.0	1.5
Dodecyl-N.N-dimethylamine-N-oxide	1%	0.0575%	2.5	2.3
Tween 20	1%	0.018%	2.5	0.8
Pentaethyleneglycolmonodecylether (C10E5)	1%	0.0775%	2.5	2.1
Octaethyleneglycolmonododecylether (C12E8)	1%	0.012%	2.5	3.7
Dimethyldecylphosphineoxide	1%	0.25%	2.5	1.3
FOSFEN 9	1%	0.13%	2.5	2.3
SucroseMonododecanoate	1%	0.04%	2.5	2.7
HEGA 11	1%	0.1375%	2.5	2.9
Tetradecyl-N.N-dimethylamine-N-oxide	1%	0.01875%	2.5	0.7
NP-40	1%	0.125%	7.0	2.7
CYMAL 5	1%	0.3%	2.5	0.7
CYMAL 7	1%	0.02475%	2.5	3.6
C-DODECAFOS	2%	0.77%	1.0	1.0
Triton X-100	1%	0.0375%	2.5	0.7
Decyl-β-D-maltopyranoside	1%	0.2175%	2.5	1.3
FOS-CHOLINE 14	1%	0.0115%	2.5	1.2
N.N-Dimethyl (3-carboxy-4-dodec-5-ene) amidopropylamine	1%	0.0178%	2.5	5.0
Dodecyl-N.N-dimethylglycine	1%	0.1025%	2.5	3.8
CYFOS 5	1%	0.375%	2.5	1.3
CYFOS 7	1%	0.055%	2.5	1.8
Dodecyl-β-D-maltopyranoside	1%	0.02%	2.3	3.8
Zwittergent 3-14	1%	0.0175%	2.5	1.2
Zwittergent 3-16	1%	0.0059%	2.5	1.6
FOS-CHOLINE 12	1%	0.1175%	2.5	1.3
FOS-CHOLINE 16	1%	0.00133%	2.5	3.2

The table lists the concentrations used for solubilization and purification of LmrA for all detergents from groups 1 to 4. Furthermore the protein yield from 5 l of culture is stated. For CTAB the protein yield is not available, because the detergent did not allow purification of LmrA by IMAC.

where necessary. For calibration of free phosphate concentrations a Na_2HPO_4 standard was used. Kinetic data were analyzed according to Michaelis–Menten kinetics:

$$\upsilon = \frac{\upsilon_{max}[S]}{K_M + [S]}$$

Here, υ denotes the reaction velocity, υ_{max} the maximal reaction velocity, S the substrate concentration and K_m the Michaelis–Menten constant.

3. Results

We screened 40 different detergents for their ability to solubilize LmrA and to facilitate its affinity purification. The used detergents cover the whole range of different chemical classes. The effect of these compounds on the activity, stability and homogeneity of LmrA were examined via measurement of ATPase activity and SEC on the day after purification as well as after 1 week of storage at 4 °C. For the interpretation of our data one has to keep in mind that co-purification of contaminating ATPases with the different detergents cannot be ruled out, especially in those systems that displayed low levels of ATPase activity. However, at least for DDM and FC-16 the values of the detergent screen agree very well with the values in Fig. 3 where the ATPase inactive E512Q mutant of LmrA (see below and [36], although references [37] reports different values) was used for background subtraction indicating that at least here, the measured ATPase activity is derived only from LmrA.

Based on the yields, homogeneity and stability of LmrA we could classify the tested detergents into four groups (Fig. 1). Group 1 contained detergents that were hardly able to solubilize LmrA from the membranes and consequently resulted in very low yields of protein (< 300 μg of protein from 5 l of culture). The SEC profiles that resulted using this group of detergents are exemplified in Fig. 2A and B for dodecyl-β-D-glucopyranoside and Big CHAP deoxy. As shown the SEC profiles of the protein samples differ substantially, indicating a very inhomogeneous group of preparations. Furthermore the protein seems to be instable based on the considerable changes in the SEC profiles upon storage for a week at 4 °C. The ATPase activity ranged from 5 (sodium dodecanoyl sarcosine) to 500 nmol min^{-1} mg^{-1} (FOS-MEA 10) when detergents of this group were used for purification. Although the SEC profiles changed during storage at 4 °C, ATPase activity remained almost the same.

In the second group, detergents can be found that are able to solubilize LmrA and allow its purification by affinity chromatography with a reasonable yield (usually>1 mg per 5 l of culture), but which resulted in a major peak in the void volume of the SEC column (molecular weight>1 MDa). This suggested aggregation of LmrA, which is depicted in Fig. 2C and D for 2-carboxy-ω-heptadecenamidopropyldimethylamine and tetradecyl-β-D-maltopyranoside,

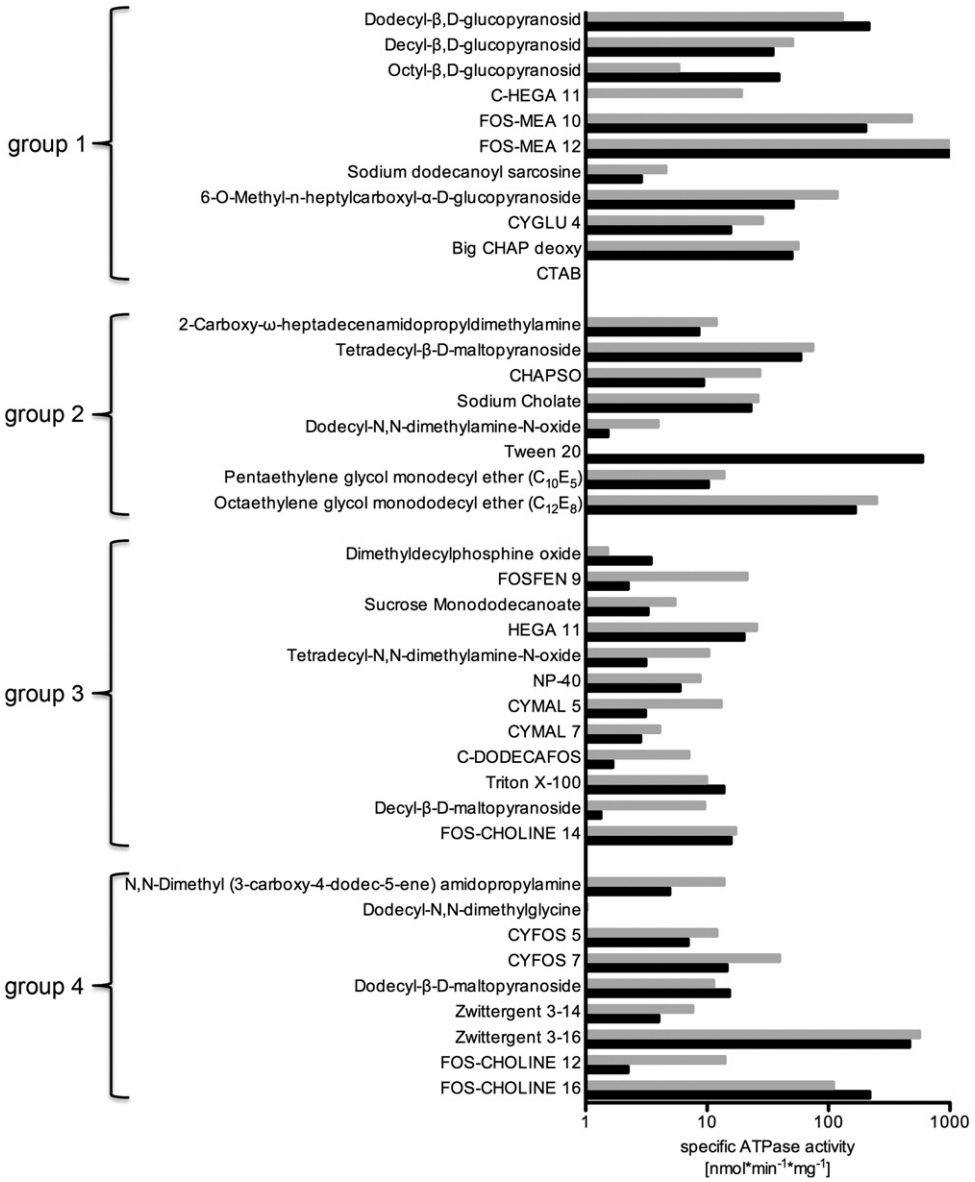

Fig. 1. ATPase activity of LmrA purified in different detergents. The specific ATPase activity of LmrA on day 1 (grey bars) and 7 (black bars) after purification with the indicated detergents are shown. The ATPase assays were performed as described in Materials and methods. The protein samples were stored at 4 °C. In the case of Tween 20, the specific ATPase activity on day 1 was not determined.

respectively. Similar to the first group of detergents, the measured ATPase activities cover a wide range from 10 nmol min^{-1} mg^{-1} for $C_{10}E_5$ up to 595 nmol min^{-1} mg^{-1} for Tween 20. Also in this group of detergents, storage at 4 °C barely influenced ATPase activity; the values remained the same except for dodecyl-N,N-dimethylamine-N-oxide and CHAPSO. Here activity decreased.

The third group consists of detergents, which resulted in yields comparable to that of the second group (usually>1 mg per 5 l of culture), but differed in the SEC profiles. The SEC analysis revealed multiple peaks indicating a substantial degree of heterogeneity as shown in Fig. 2E and F for Triton X-100 and CYMAL 7, respectively. For CYMAL 7, SEC analysis resulted in two peaks of similar height. The first peak (void volume) corresponded most likely to aggregated protein whereas the second peak had a molecular weight of approximately 450 kDa. In contrast SEC analysis of LmrA purified in Triton X-100 revealed no aggregated protein. Instead its analysis resulted in two peaks with molecular weights of approximately 450 and 150 kDa, the latter of which—keeping the limitations of SEC on membrane proteins in mind—might resemble dimeric LmrA in a detergent micelle. In terms of ATPase activity this group of detergents was more homogeneous than the previous groups with similar ATPase activities for all members.

The fourth group contained nine different detergents that resulted in good yields of LmrA (>1.5 mg per 5 l of culture) and only one major peak besides a minor peak in the void volume in SEC analysis (see Figs. 2G, H, 3B and E), which pointed to a certain degree of homogeneity. There was some variation among the detergents in the relative height and number of minor peaks. In case of Zwittergent 3-14, Zwittergent 3-16 and CYFOS 7 the peak in the void volume was smaller relative to the major peak compared to dodecyl-N,N-dimethylglycine. CYFOS 7 and more pronounced Zwittergent 3-14 gave rise to a small additional peak corresponding to a molecular weight of approximately 70 kDa. Upon storage at 4 °C all samples showed a reduction of the peak in the void volume relative to the main peak, which was most pronounced with Zwittergent 3-16. With respect to the ATPase activities, results were comparable to the other detergent groups, but were also very heterogeneous. No activity was detectable for dodecyl-N,N-dimethylglycine, low activity for Zwittergent 3-14 (8 nmol min^{-1} mg^{-1}), intermediate activities for CYFOS 7 (40 nmol

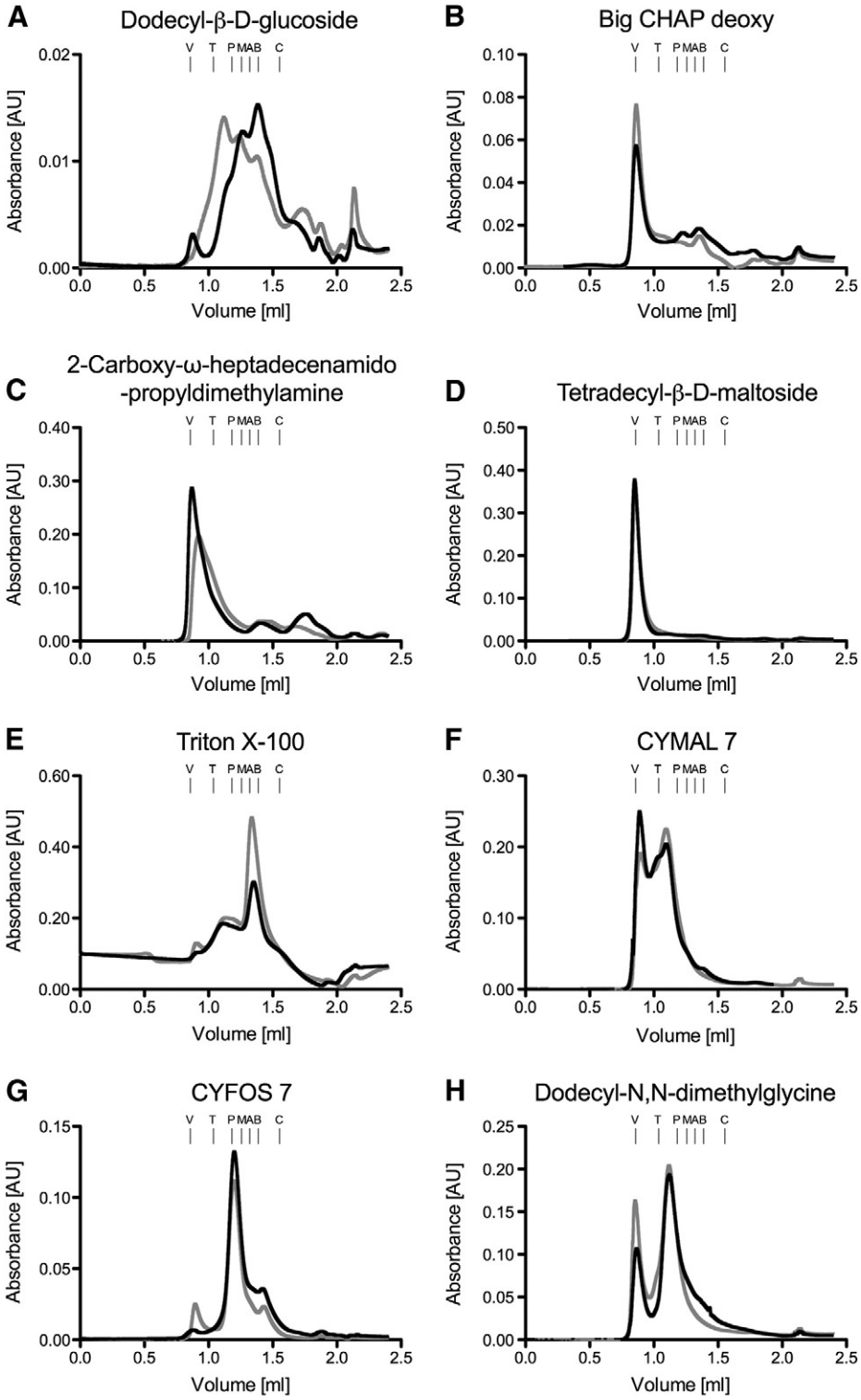

Fig. 2. SEC profiles of typical LmrA preparations obtained with detergents from group one to four. The figure shows SEC profiles of LmrA preparations on day 1 (grey lines) and 7 (black lines) after purification. Between the SEC analysis the samples were stored at 4 °C. Furthermore the elution volumes of soluble marker proteins with the following molecular weights (in kDa) as well as the void volume (V) of a Superose 12 column are indicated: C 29, B 66, A 150, M 200, P 443, T 669.

min^{-1} mg^{-1}) and one of the highest activities for Zwittergent 3-16 (560 nmol min^{-1} mg^{-1}).

Taking the SEC analysis and the ATPase activities of all preparations into account, the best results were obtained for FC-16. The SEC profile of this preparation on the day after purification resulted in one major peak at approximately 400 kDa with only a small additional peak in the void volume (Fig. 3E). Upon storage for a week at 4 °C, the elution profile of the SEC analysis remained basically unchanged. Furthermore, the ATPase activity (110 nmol mg^{-1} min^{-1}) is one of the highest measured in this detergent screen with only Zwittergent 3-16, Tween 20 and FOS-MEA 10 and 12 displaying higher activities.

Based on the results of the detergent screen we have chosen FC-16 and DDM for a more detailed characterization of LmrA. With both detergents high yields of LmrA ($\approx$ 0.8 mg/l culture), only one major

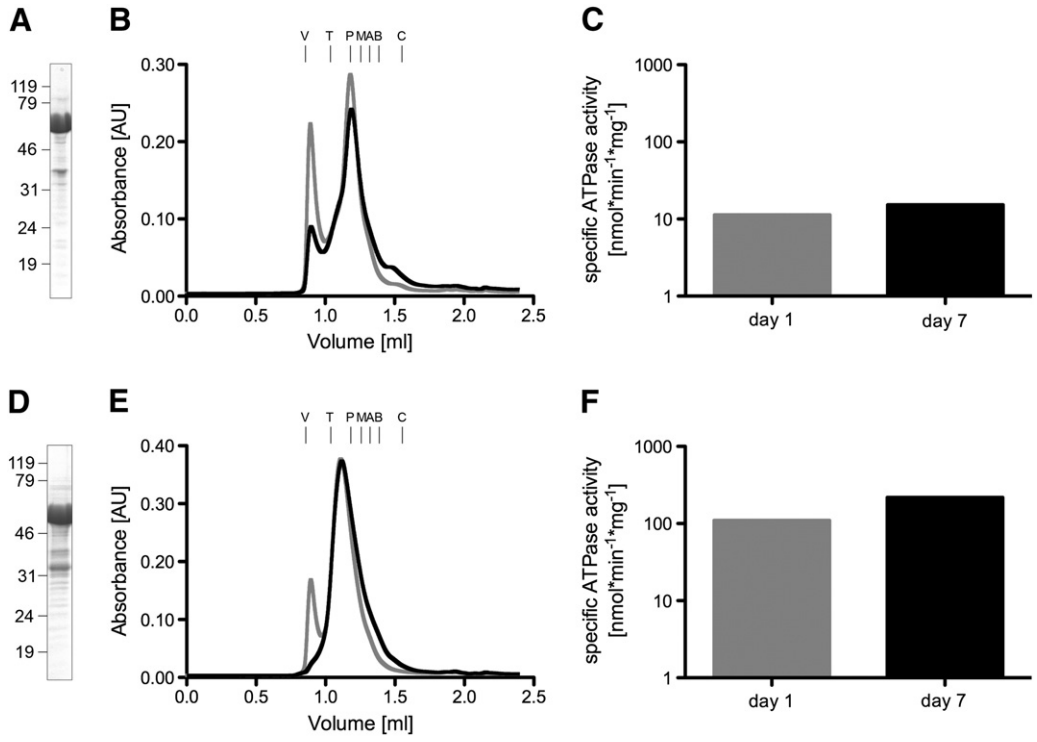

Fig. 3. Characterization of LmrA purified with Dodecyl-β-D-maltoside and FOS-CHOLINE-16. (A) Coomassie brilliant blue stained SDS-PAGE of LmrA in DDM after IMAC chromatography. The positions of the molecular weight markers are shown on the left (in kDa). (B) Size exclusion chromatogram of LmrA in DDM on day 1 (grey lines) and 7 (black lines). (C) Specific ATPase activity of LmrA in DDM on day 1 (grey bar) and 7 (black bar) after purification. (D) Coomassie brilliant blue stained SDS-PAGE of LmrA in FC-16 after IMAC. Molecular weight markers are indicated on the left (M_W in kDa). (E) Analysis of LmrA in FC-16 by SEC on day 1 (grey lines) and 7 (black lines). (F) Specific ATPase activity of LmrA in FC-16 on day 1 (grey bar) and 7 (black bar). Storage of the protein samples between the experiments was performed at 4 °C.

peak in the SEC analysis and a measurable ATPase activity in detergent solution (Fig. 3A, C, D and F) were obtained. Furthermore, LmrA seems to be stable for at least a week as evident by the similar SEC profiles and ATPase activities upon storage at 4 °C (Fig. 3B, C, E and F). Although, we obtained a stable and homogeneous LmrA sample (in view of the SEC profiles) with both detergents, the specific ATPase activities varied considerably. In FC-16, LmrA displayed a specific ATPase activity of 110 nmol min^{-1} mg^{-1} whereas a specific activity of only 10 nmol min^{-1} mg^{-1} was measured in DDM at an ATP concentration of 1 mM. To further investigate this difference, the K_m and V_{max} values of ATP hydrolysis of LmrA in the solubilized state were determined by Michaelis–Menten kinetic analysis (Fig. 4A). Purified in FC-16, LmrA had a V_{max} of 125.6 ± 8.9 nmol mg^{-1} min^{-1} and a K_m value of 0.4 ± 0.1 mM for ATP, that leads to a turnover number of 8.2 ± 0.6 min^{-1}. In contrast LmrA purified in DDM resulted in a V_{max} of 26.8 ± 3.9 nmol mg^{-1} min^{-1} and a K_m of 1.8 ± 0.6 mM corresponding to a k_{cat} value of 1.7 ± 0.3 min^{-1} for ATP, while the inactive E512Q mutant of LmrA (see [36], although references [37] reports different values) showed basically no ATPase activity (V_{max} < 2 nmol min^{-1} mg^{-1}) in either FC-16 or DDM and was used for background subtraction. Therefore, the ability of LmrA to hydrolyze ATP in detergent solution is impaired in DDM when compared to FC-16. Blue native page electrophoresis (data not shown) indicated similar amounts of homodimer in both samples ruling out dimer dissociation as possible explanation for the observed differences. To further address the influence of the two detergents on ATPase activity, we analyzed the ATPase activity in the presence of a substrate of LmrA, rhodamine 123 [38]. In case of LmrA purified in FC-16, the specific ATPase activity could be stimulated twofold by the addition of low concentrations of rhodamine 123 (Fig. 5A), which decreased to basal levels at higher substrate concentrations [39]. In contrast to that LmrA purified in DDM showed low ATPase activity that could not be stimulated by rhodamine 123 (Fig. 5A).

To characterize LmrA in its natural environment—the lipid bilayer—we reconstituted the purified protein into preformed liposomes of *E. coli* total lipids using either FC-16 or DDM and measured its ATPase activity (Fig. 4B). For LmrA purified and reconstituted in DDM the Michaelis–Menten kinetics fit resulted in a V_{max} of 128.4 ± 6.8 nmol mg^{-1} min^{-1} and a K_m value of 0.6 ± 0.1 mM resulting in a k_{cat} value of 8.3 ± 0.4 min^{-1} for ATP. In contrast, LmrA purified and reconstituted in FC-16 resulted in a V_{max} of 60.3 ± 8.8 nmol mg^{-1} min^{-1} and a K_m value of 3.0 ± 0.8 mM corresponding to a k_{cat} value of 3.4 ± 0.5 min^{-1} for ATP, while the control with the inactive E512Q mutant of LmrA displayed no detectable ATPase for both reconstitution procedures (DDM or FC-16) and was again used for background subtraction. For comparison the kinetic of ATP hydrolysis of LmrA in *L. lactis* membrane vesicles was performed (background subtraction with membrane vesicles of the inactive E512Q mutant of LmrA) and resulted in a V_{max} of 92.0 ± 4.1 nmol mg^{-1} min^{-1} and a K_m value of 0.7 ± 0.1 mM for ATP (Fig. 4C) which is similar to the values obtained for DDM-reconstituted LmrA liposomes. In contrast to the experiments with solubilized LmrA in DDM (Fig. 5A), rhodamine 123 stimulated the basal ATPase activity of DDM reconstituted LmrA two-fold at a concentration of 50 nM rhodamine 123 (Fig. 5B). This stimulatory effect on the ATPase activity of LmrA is less pronounced at higher substrate concentrations. In contrast FC-16 reconstituted LmrA displayed only a slight inhibition of the basal ATPase activity at high (> 600 nM) rhodamine 123 concentrations (Fig. 5B).

4. Discussion

Here, we have reported a comprehensive detergent screen for the ABC-transporter LmrA that visualizes the different outcome of detergent solubilization of membrane proteins and reports data on this important yet underrepresented field of membrane protein research in the literature. In contrast to many other detergent screens described so far

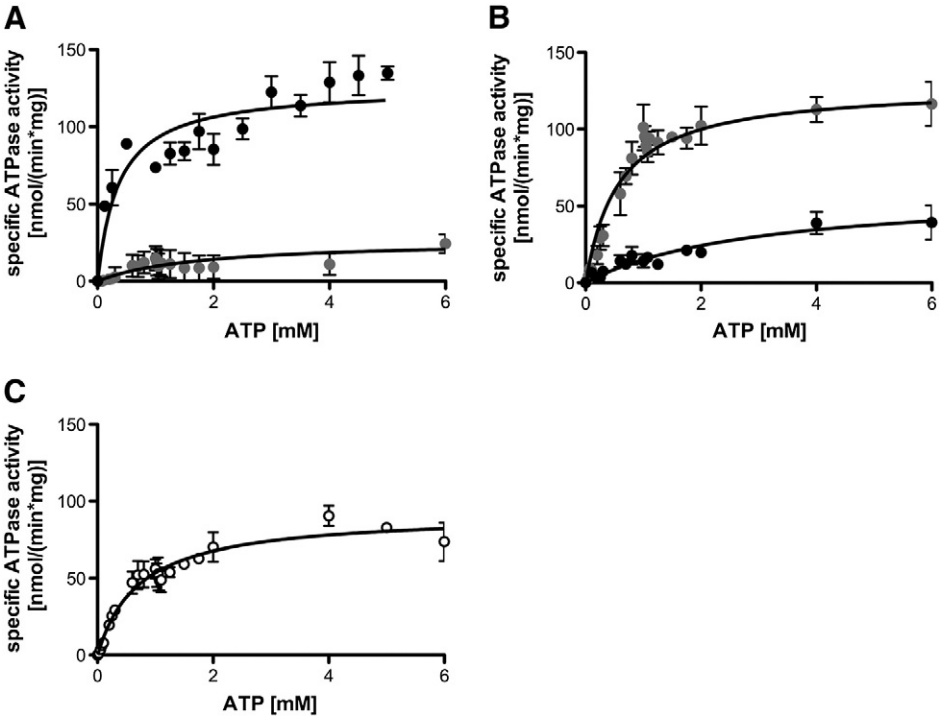

Fig. 4. Kinetics of ATP hydrolysis of LmrA. (A) Specific ATPase activity of purified LmrA in buffer 4 with 5 mM $MgCl_2$ supplemented with DDM (grey) or FC-16 (black). (B) Specific ATPase activity of LmrA reconstituted with DDM (grey) or FC-16 (black). (C) Specific ATPase activity of LmrA enriched membrane vesicles from *L. lactis*, in this case the total protein in the vesicles is used for the calculation of the ATPase activity. The results of the ATPase inactive E512Q mutant of LmrA were subtracted in all measurements as background.

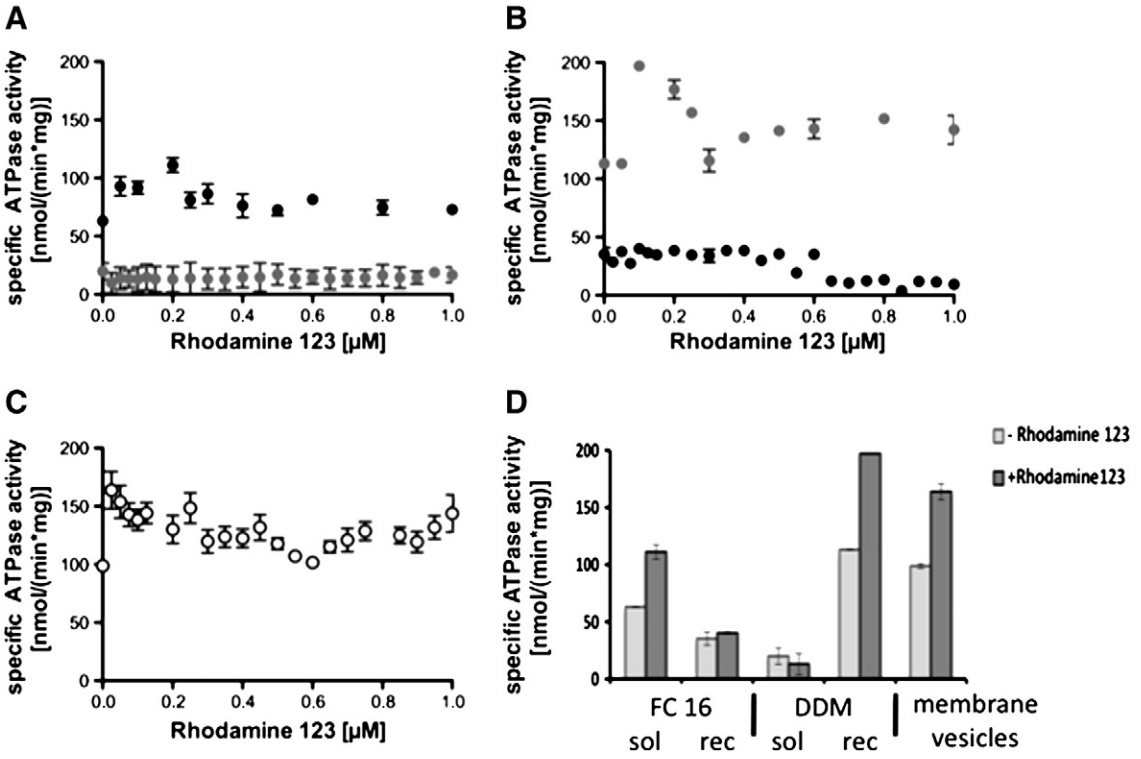

Fig. 5. Specific ATPase activity of LmrA in presence of substrate. (A) Specific ATPase activity of LmrA purified in DDM (grey) or FC-16 (black) in buffer 4 supplemented with 5 mM $MgCl_2$, the respective detergent and increasing rhodamine 123 concentrations. (B) Specific ATPase activity of reconstituted LmrA purified with DDM (grey) or FC-16 (black) with increasing rhodamine 123 concentrations (C) Specific ATPase activity of *L. lactis* membrane vesicles from cells overexpressing LmrA with increasing rhodamine 123 concentrations (D) Overview of the substrate stimulated ATPase activity of LmrA in the presence of rhodamine 123 (grey bars) in comparison to the basal ATPase activity in the absence of rhodamine 123 (white bars). Concentrations of rhodamine 123 shown correspond to those, which displayed the highest degree of stimulation (200 nM for FC 16 solubilized or reconstituted LmrA, 100 nM for DDM solubilized or reconstituted LmrA and 50 nM for membrane vesicles).

(e.g. [5–9]), this study not only evaluated the solubilization efficiency, but also analyzed structural and functional properties of LmrA directly after purification and upon storage at 4 °C. The screen revealed that seven out of the nine best-suited detergents were zwitterionic. Interestingly three of these detergents (Zwittergent 3-14, Zwittergent 3-16 and FC-16) contained unusually long C14 to C16 acyl chains. This was not expected, because so far non-ionic detergents of intermediary size usually proved to be superior for the purification of membrane proteins (for a review see for example [11]). Nevertheless, there are examples in the literature where zwitterionic detergents were successfully used, especially with ABC-transporters [5,40]. Two major reasons should be considered to explain the preference of LmrA for zwitterionic detergents especially the FOS-CHOLINE detergents. (I) Zwitterionic detergents might stabilize the transmembrane domains of the protein, because their micelles mimic the lipid bilayer. The detergents of the phosphocholine class are not only favored in NMR studies but are also used as starting points for the design of new detergents [41]. (II) The zwitterionic detergents and here especially FC-16 might have a greater ability to co-purify lipids together with the protein, which in case of the 5-HT_{1A}-receptor from sheep brain [13] and the sarcoplasmic reticulum Ca^{2+}-ATPase [16] enhanced stability and catalytic activity.

The detailed analysis of LmrA in DDM and FC-16 revealed two ATPase active samples, the FC16-solubilized and DDM-reconstituted LmrA. In both cases, we obtained a similar k_{cat} value of around 8 min^{-1}. Two major questions arise from our results: 1) Why is the ATPase activity of LmrA solubilized in DDM so low? 2) Why is the ATPase activity reduced after reconstitution in case of FC-16? Unfortunately, we cannot answer these questions at this stage of our work. One possible reason for the impaired activity in DDM might be a direct influence of the detergent. For human Pgp, it has been demonstrated that some detergents are substrates [42]. Remarkably, in most purification protocols of LmrA [43,44] DDM was used, and there were even higher values for the ATPase activity for solubilized LmrA reported than those measured here. Therefore, incubation time, protein to detergent ratio as well as the purity grade of the detergent used may also have an impact on the activity of LmrA and would explain the discrepancy between our results and those reported. Since lipids can influence the activity of ABC transporters [45,46] it may well be that in contrast to DDM, FC-16 ensures the copurification of a lipid that is necessary for the activity of LmrA, so that activity of the DDM sample can only be obtained after reconstitution. This would suggest that LmrA needs a certain lipid to display its activity. In line with this model, specific binding of cardiolipin to LmrA has been shown by mass spectrometry [44]. Furthermore, it should be mentioned that our measured V_{max} value is smaller than the ATPase activity (230 nmol min^{-1} mg^{-1}) reported in Ref. [36] for reconstituted LmrA. This might be due to determination of the activity, because we can only consider the total protein and not the effective protein amount, i.e. those LmrA molecules reconstituted in a right-site-out fashion (NBDs accessible for the ATP). This can also explain the second question, concerning the reduced activity of the reconstituted LmrA in case of FC-16 to a certain extent. This would suggest that the percentage of LmrA with the NBDs facing the lumen of the proteolipomes is higher in the FC-16 sample than in the DDM sample. Consequently, we would then expect that the distribution of ATPase activity with the different rhodamine 123 concentrations would match that of the reconstituted DDM sample with only lower values, but this is not the case. Nevertheless, the ability of the protein to show a response to the presence of a substrate is one important factor to prove functional reconstitution in the field of MDR ABC transporters [47].

5. Conclusions

In summary, our study illustrates the key importance of detergents in the solubilization and purification of the ABC transporter LmrA and adds additional information to this important aspect of membrane protein research. Our study revealed the preference of LmrA for zwitterionic detergents. Interestingly further studies on LmrA in DDM and FC-16 showed that FC-16 solubilized LmrA displayed substrate-stimulated ATPase activity while this was not the case for DDM. Unexpectedly, an opposite behavior was observed for the reconstituted protein. Here, DDM-reconstituted LmrA could be stimulated by substrate, whereas FC-16 reconstituted protein showed a low ATPase activity and a minor substrate inhibition. These findings emphasize the importance of detergents for the study of LmrA both in the solubilized and in the reconstituted state, although further investigations will be necessary to explain the differences observed for detergent solubilized and reconstituted LmrA.

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